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In Vivo Formation of Hybrid Toxins Comprising Shiga Toxin and the Shiga-Like Toxins and Role of the B Subunit in Localization and Cytotoxic Activity

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Shiga toxin, Shiga-like toxin I (SLT-I) and Shiga-like toxin II (SLT-II) are cell-associated cytotoxins that kill both Vero cells and HeLa cells, whereas Shiga-like toxin II variant (SLT-IIv) is an extracellular cytotoxin that is more cytotoxic for Vero cells than for HeLa cells. The basis for these differences in cytotoxin localization and host cell specificity were examined in this study. The A and B subunit genes of Shiga toxin and the SLTs were recombined by two methods so that hybrid toxins would be formed in vivo. Complementation of heterologous subunits was accomplished by cloning the individual A and B subunit genes of SLT-I, SLT-II, and SLT-IIv on plasmid vectors of different incompatibility groups so that they could be maintained in double transformants of *Escherichia coli*. In addition, six operon fusions were constructed so that the A and B subunit genes of Shiga toxin, SLT-II, and SLT-IIv could be expressed as a single operon. The activities of the hybrid cytotoxins were assessed in three ways: (i) level of cytotoxicity, (ii) ratio of HeLa to Vero cell cytotoxicity, and (iii) ratio of extracellular to cell-associated cytotoxicity. Neither the A subunit of Shiga toxin nor SLT-I associated with a heterologous B subunit to form an active cytotoxin. However, in all other cases the hybrid molecules formed by subunit complementation or operon fusion were cytotoxic. Furthermore, the cytotoxic specificity and localization of the hybrid cytotoxins always corresponded to the activities of the native toxin possessing the same B subunit.

The Shiga-like cytotoxins (SLTs; also called Verotoxins) have been categorized into two antigenically distinct types. The first group includes Shiga toxin and SLT-I, which are neutralized by anti-Shiga toxin and monoclonal antibodies to the SLT-I B subunit (24, 25, 33). These toxins are now referred to as Shiga toxin/SLT-I on the basis of the recent finding that the *stx* and *slt*-I nucleotide sequences are essentially identical (13, 33). Indeed, there are only three nucleotide differences between *stx* and *slt*-I, which result in an identical deduced amino acid sequence except at positions 45 of the A subunit (Shiga toxin has a threonine, whereas SLT-I has a serine at that position). The second group of SLTs includes SLT-II and SLT-IIv, both of which are neutralized by polyclonal antisera against SLT-II (21, 27) but not by anti-Shiga toxin. SLT-IIv is classified as a variant of SLT-II because it is significantly more cytotoxic for Vero cells than for HeLa cells (21). SLT-I and SLT-II are produced by enterohemorrhagic *Escherichia coli* that cause hemorrhagic colitis and the hemolytic uremic syndrome in humans (21, 35, 39; S. M. Scotland, H. R. Smith, G. A. Willshaw, and B. Rowe, Letter, Lancet ii:216, 1983), while SLT-IIv is produced by *E. coli* isolated from swine with edema disease (21).

The mechanisms of action of Shiga toxin, SLT-I, SLT-II, SLT-IIv, and the plant toxin ricin are the same (6, 7, 31). These toxins are *N*-glycosidases that cleave a specific adenine residue from the 28S subunit of eucaryotic rRNA, which results in the inhibition of protein synthesis. These bipartite toxins consist of an A subunit, which is responsible for the enzymatic activity, noncovalently linked to multiple B subunits that are responsible for binding to cellular receptors (4, 26, 28). The eucaryotic receptor to which the B subunits of Shiga toxin, SLT-I, and SLT-II bind is a galactose-α1,4-galactose-containing glycolipid designated Gb_3 (12, 16–18, 36). SLT-IIv does not bind to a Gb_3 analog, galactose-α1,4-galactose conjugated to bovine serum albumin, which suggests that SLT-IIv may bind a different cellular receptor than the other members of the Shiga toxin family do (10, 38).

Individual subunits of SLT-I and SLT-II can assemble to form fully cytotoxic hybrid molecules in vitro, as recently demonstrated by Ito et al. (11) with purified A and B subunits of the toxins. In the study presented here, heterologous cytotoxins comprised of the individual subunits of Shiga toxin/SLT-I, SLT-II, and SLT-IIv were created in vivo by complementation and by operon fusion. The relative importance of the A and B subunits for determining cell specificity and extracellular localization of holotoxin was assessed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Media, enzymes, biochemicals, and radionuclides. Luria broth or Luria broth agar (19) was used for routine culturing of bacteria. Where indicated, media were supplemented with antibiotics (Sigma Chemical Co., St. Louis, Mo.) at the following concentrations: ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), kanamycin (50 μg/ml), and tetracycline (12.5 μg/ml). Agarose for DNA electrophoresis was purchased from International Biotechnologies, Inc. (New Haven, Conn.).

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). DNA poly-

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TABLE 1. Bacterial strains, plasmids, and bacteriophages used in this work

Strain or plasmid	Description or genotype[a]	Reference or source
Strains		
E. coli HB101	F⁻ *hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44* λ^-	32
E. coli DH5α	F′ ϕ80d*lacZ* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA relA1*	32
E. coli JM109	*endA1 gyrA96 thi hsdR17 supE44 relA1 traD36* Δ(*lac proAB*)/F′ *proAB lacI*^{q}Z M15	Bethesda Research Laboratories, Gaithersburg, Md.
Plasmids		
pBR329	Tc^r, Ap^r, Cm^r; replicon ColE1	3
M13 mp18 and mp19	Sequencing vectors	Boehringer Mannheim
pACYC184	Cm^r Tc^r, replicon P15A	2
pBluescript SK	Ap^r, expression vector	Stratagene
pBluescribe	Ap^r, expression vector	Stratagene
pJN25	pBR328 with *slt*-I; Ap^r	22
pNAS10	pBR329 with *stx*; Ap^r	33
pNN103	pBR328 with *slt*-II; Ap^r	23
pMJ100	pBluescribe with *slt*-II; Ap^r	This study
pDLW5	pBR329 with *slt*-IIv; Ap^r	This study
pDLW5.3	pDLW5 with *Eco*RI 5′ to *slt*-IIvA	This study
pDLW5.104	pDLW5 with *Eco*RI in *slt*-IIv A	This study
pMJ153	pNAS10 with created *Eco*RV site; Ap^r	This study
pMJ330	pMJ100 with created *Hpa*I site; Ap^r	This study
pDLW5.321	pDLW5.3 with created *Hpa*I site; Ap^r	This study
pDW8	pBR328 with *slt*-I; mini-Mu in B subunit gene; Km^r, Ap^r	This study
pMJ331	pBluescribe with *slt*-II A from pMJ330; Ap^r	This study
pDLW101	pACYC184 with *slt*-I A; Cm^r	This study
pJN26	pBR328 with *slt*-I B; Ap^r	22
pDLW102	pACYC184 with *slt*-II A; Cm^r	This study
pDLW103	pBluescript with *slt*-II B; Ap^r	This study
pDLW104	pACYC184 with *slt*II-v A; Tc^r	This study
pDLW105	pBluescript with *slt*II-v B; Ap^r	This study
pFUS1	ϕ(*slt*-IIv A-*slt*-II B) in pBR329; Ap^r	This study
pFUS2	ϕ(*slt*-II A-*slt*-IIv B) in pBR329; Ap^r	This study
pFUS3	ϕ(*stx* A-*slt*-II B) in pBR329; Ap^r	This study
pFUS4	ϕ(*slt*-II A-*stx* B) in pBR329; Ap^r	This study
pFUS5	ϕ(*stx* A-*slt*-IIv B) in pBR329; Ap^r	This study
pFUS6	ϕ(*slt*-IIv A-*stx* B) in pBR329; Ap^r	This study

[a] Abbreviations: Tc^r, Ap^r, Cm^r, and Km^r, resistance to tetracycline, ampicillin, chloramphenicol, and kanamycin, respectively. *stx*, Shiga toxin operon; *slt*, Shiga-like toxin operon; A, A subunit gene; B, B subunit gene (of *stx* or *slt*); ϕ, operon fusion.

merase I (Klenow fragment), calf intestinal alkaline phosphatase, T4 DNA ligase, and DNA kinase were purchased from Boehringer Mannheim. Sequenase DNA sequencing kit was purchased from U.S. Biochemicals Corp., Cleveland, Ohio. The DNA mutagenesis kit was purchased from Bio-Rad Laboratories (Richmond, Calif.).

Radionuclides were purchased either from Dupont, NEN Research Products (Boston, Mass.) or from Amersham Corp. (Arlington Heights, Ill.).

Cytotoxicity and neutralization assays. Microcytotoxicity assays were done on Vero and HeLa cells according to modifications described previously (20) of the published methods of Gentry and Dalrymple (9). The amount of toxin contained in the last 10-fold dilution of the sample in which greater than or equal to 50% of the HeLa or Vero cells detached from the plastic, as assessed by A_{620}, was considered to be the 50% cytotoxic dose (CD_{50}).

Toxin neutralization assays with polyclonal anti-SLT-II and anti-Shiga toxin were done as described previously (24).

Cloning and DNA preparation. Routine cloning and preparation of plasmid DNA procedures were done as described by Maniatis et al. (19). When appropriate, individual restriction fragments were isolated by electroelution by following the instructions supplied by the manufacturer of the electroeluter (International Biotechnologies).

Nucleotide sequence analysis. Either the M13 universal primer (New England BioLabs, Inc., Beverly, Mass.) or synthetic oligonucleotides prepared with a model 380A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) were used as primers in the dideoxy-chain terminator method (1, 29, 30), with a Sequenase kit (U.S. Biochemicals Corp.), by the method provided by the supplier of the kit.

Oligonucleotide-directed site-specific mutagenesis. The procedure for oligonucleotide-directed site-specific mutagenesis was modified from the protocol of Zoller and Smith (40). Single-stranded M13 DNA containing the selected insert was purified from *E. coli* CJ236 by using standard procedures supplied in the kit (Bio-Rad). A synthetic oligonucleotide (25- to 30-mer) with one or three nucleotide difference(s) from the wild-type gene was annealed to the uracil-containing, single-stranded DNA template. The second strand was then synthesized by using DNA polymerase 1 (Klenow fragment) and deoxynucleoside triphosphates (dATP, dCTP, dGTP, and TTP; Sigma). The 3′ and 5′ ends of the newly synthesized strand were ligated with T4 DNA ligase. The ligation mixtures were used to transfect *E. coli* JM109, and the transfectants were plated for plaque detection by using a 1% Luria broth agar overlay containing approximately 2×10^8 logarithmic-phase *E. coli* JM109 (32). Single-stranded phage DNAs were prepared from selected transfectants, and the nucleotide sequences were determined. Isolates containing the desired mutation were plaque purified and rese-

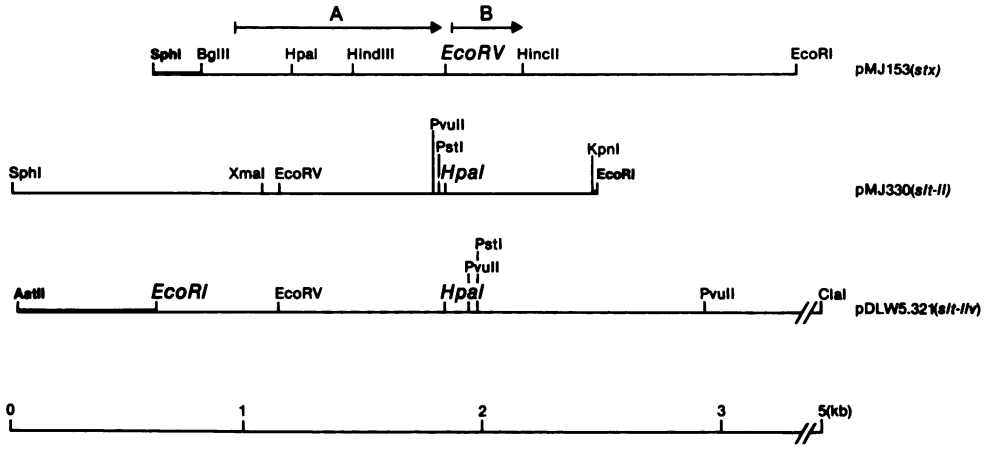

FIG. 1. Physical maps of the modified *stx*, *slt*-II, and *slt*-IIv operons cloned in plasmids pMJ153, pMJ330, and pDLW5.321, respectively. The *Eco*RI restriction site created by linker insertion and the *Eco*RV and *Hpa*I sites created by oligonucleotide-directed site-specific mutagenesis are indicated in italics. Vector DNA is shown by heavy lines, and restriction sites in vector DNA are indicated in bold type. The location and orientation of the A and B subunit structural genes are shown above the restriction maps. Distances are given in kilobases (kb).

quenced to ensure the fidelity of the mutation. Double-stranded DNA was prepared from the transfectants, and the desired fragments were used in plasmid construction.

Plasmid construction. As a first step in the construction of plasmids carrying the individual A or B SLT subunit gene, unique restriction sites were created in plasmid pDLW5 (38; contains intact *slt*-IIv operon) to facilitate subcloning individual restriction fragments. Novel *Eco*RI restriction sites were inserted within the *Rsa*I sites of *slt*-IIv in pDLW5 (Table 1) by using 8-base-pair *Eco*RI linkers (Pharmacia Fine Chemicals, Piscataway, N.J.). Plasmid pDLW5 was linearized by partial digestion with *Rsa*I (0.1 U of *Rsa*I per μg of DNA at 37°C for 10 min). The linearized DNA was ligated to 5'-phosphorylated *Eco*RI linkers, digested to completion with *Eco*RI, and subjected to electrophoresis on a 0.7% preparative agarose gel. Linear plasmid DNA was excised from the gel, isolated by electroelution, ethanol precipitated, recircularized by ligation, and transformed into *E. coli* HB101. In plasmid pDLW5.3, an *Eco*RI site was inserted 5' to the *slt*-IIv operon, and in pDLW5.104, the *Eco*RI site was inserted within the *slt*-IIv A gene between nucleotide positions 1006 and 1007 (37). The insertion in pDLW5.3 did not affect the expression of SLT-IIv, as determined by a Vero cell cytotoxicity assay, whereas the insertion in pDLW5.104 resulted in the complete loss of cytotoxicity (data not shown). Clones producing high levels of cytotoxicity were maintained under BL3+EK1 containment (8).

Next, single *Hpa*I or *Eco*RV restriction sites were created in the 12 to 15 nucleotide gaps between the A and B subunit genes of *stx*, *slt*-II, and *slt*-IIv by using oligonucleotide-directed site-specific mutagenesis (Fig. 1; 13, 33, 38). The specific restriction site created in each operon was selected for the following reasons: (i) the restriction site did not naturally exist within the operon; (ii) no more than three nucleotide changes were required for the creation of any site; (iii) digestion at all of the created sites generated blunt-ended DNA fragments which were compatible for the subsequent construction of operon fusions; and (iv) the changes did not alter the putative ribosome-binding sequences nor any coding sequences for the B subunit genes. An *Eco*RV site was created in the 12 nucleotide gap between the A and B subunit genes of *stx* to create pMJ153 (Fig. 1; 33) by changing the following nucleotides: guanine-1113 to adenine, adenine-1116 to thymine, and adenine-1117 to cytosine. Plasmid pMJ100 was constructed by cloning the 2.3-kilobase (kb) *Sph*I-*Eco*RI fragment of *slt*-II from pNN103 (23) into the expression vector pBluescribe. To construct pMJ330, an *Hpa*I site was created in pMJ100 by changing a guanine to a cytosine in the gap between the A and B subunit genes of *slt*-II at position 1204 (13) and in the *slt*-IIv operon at position 1212 to construct pDLW5.321 (38; Fig. 1 and Table 1). These sequence changes did not affect the expression of the toxin genes, as determined by Vero cell cytotoxicity assays (data not shown).

The third step in the construction of plasmids for the complementation studies was to clone the A and B subunit genes of each cytotoxin into plasmids of different incompatibility groups (Fig. 2). This was done to ensure the stable cotransformation of a single cell with two plasmids. Plasmids pDW8, pMJ331, and pDLW5 were used to construct A subunit subclones of SLT-I, SLT-II, and SLT-IIv, respectively. Plasmid pDW8 (37) carries an operon fusion which was derived by the insertion of a transposable mini-Mu *lac* element into the *slt*-I B gene of plasmid pJN25 (22). Plasmid pMJ331, which carries the individual *slt*-II A subunit gene, was constructed by cloning the 1.8-kb *Sph*I-*Hpa*I restriction fragment from pMJ330 (Fig. 1) into the *Sph*I and *Hinc*II sites of the pBluescript vector. The individual A subunit genes of Shiga toxin/SLT-I, SLT-II, and SLT-IIv were then cloned into pACYC184, which has a P15A replicon (2). The B subunit genes of the SLTs were cloned into either pBR328 or pBluescript SK, both of which have a ColE1 replicon (3). In previous studies, immunoprecipitation and colony blot assays demonstrated that pJN26 (*slt*-I B subunit gene in pBR328) produces SLT-I B subunit (22, 37). The *slt*-IIv and *slt*-II B subunit genes were cloned into the expression vector pBluescript SK; hence, the genes were transcribed from a vector promoter.

Operon fusions. Operon fusions containing the heterologous A and B subunit genes of *stx*, *slt*-II, and *slt*-IIv were constructed. The A and B subunit genes from plasmids pMJ153 (*stx*), pMJ330 (*slt*-II), and pDLW5.321 (*slt*-IIv) were isolated on the appropriate restriction fragments. Each fragment encoding an A subunit was then ligated to a fragment encoding a different B subunit, and these six operon fusions were cloned into plasmid vector pBR329. The individual restriction fragments used for creating the fusions were (Fig. 1): (i) the 1.3-kb *Sph*I-*Eco*RV (*stx* A) and 1.4-kb *Eco*RV-

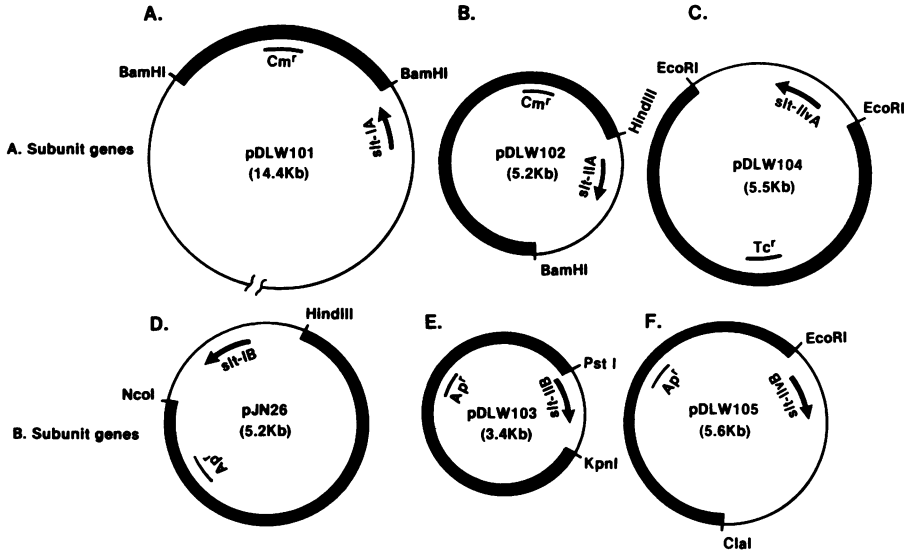

FIG. 2. Physical maps of the plasmids carrying the individual A and B subunit genes used in complementation studies. To ensure the stable cotransformation of a single cell with two plasmids, one carrying an individual A subunit gene and the other carrying an individual B subunit gene, the A and B subunit genes of each cytotoxin were cloned into plasmid vectors of different incompatibility groups. The restriction sites which flank the cloned inserts are indicated on each plasmid. Vector DNA is indicated by bold lines (vectors: [A through C], pACYC184; [D], pBR328; [E and F], pBluescript SK). The source of the cloned insert DNA was: (A), pDW8; (B), pMJ331; (C), pDLW5.3; (D), pJN26 (Newland); (E), pNN103; (F), pDLW5.104. The approximate locations of the subunit genes within the inserts are indicated by arrows. The abbreviations used are given in Table 1.

*Eco*RI (*stx* B) fragments from pMJ153; (ii) the 1.8-kb *Sph*I-*Hpa*I (*slt*-II A) and 0.5-kb *Hpa*I-*Eco*RI fragment of pMJ330 (*slt*-II B); and, (iii) the 1.7-kb *Aat*II-*Hpa*I (*slt*-IIv A) and 2.3-kb *Hpa*I-*Cla*I (*slt*-IIv B) fragments from pDLW5.321. The operon fusions, designated pFUS1-6, which are described in Table 1, were confirmed by nucleotide sequence analysis.

Dot blot analysis. A dot blot assay (modified from the colony-enzyme-linked immunosorbent assay of Strockbine et al.; 34) was used to assess whether SLT-I B or SLT-II B subunit-specific monoclonal antibodies could recognize the homologous subunit when associated with a heterologous A subunit. Bacterial cultures (250 ml) were disrupted by sonication. The resulting lysates were then clarified by centrifugation and concentrated by precipitation with ammonium sulfate (24). The precipitates were dialyzed against phosphate-buffered saline (0.15 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.6), and the volumes were brought to 1 ml with phosphate-buffered saline. Portions (5, 10, and 30 μl) of the ammonium sulfate-precipitated samples were spotted on nitrocellulose membranes (Millipore Corp., Bedford, Mass.). Immunochemical staining of the blots was accomplished as follows: (i) the blots were blocked with 1% (wt/vol) bovine serum albumin (Sigma) in Tris-buffered saline (20 mM Tris hydrochloride, 500 mM NaCl; pH 7.5) for 1 h at room temperature with gentle agitation; (ii) the blots were incubated for 1 h at room temperature with monoclonal antibody specific for the SLT-II B subunit (5; 1:5,000 dilution of mouse ascitic fluid in Tris-buffered saline–1% bovine serum albumin), and unbound antibody was removed by five 5-min washes with 0.05% Tween 20 (Sigma) in Tris-buffered saline at room temperature; (iii) the blots were incubated with an alkaline phosphatase-linked goat anti-mouse IgG (Stratagene; 1:5,000 dilution in Tris-buffered saline–1% bovine serum albumin), and the excess antibody was removed as described above; and (iv) the blots were developed with the substrate (0.3 mg of nitroblue tetrazolium per ml and 0.15 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml in 100 mM Tris hydrochloride, 100 mM NaCl, and 5 mM $MgCl_2$) in the dark for 15 min.

Radioiodination and immunoprecipitation. *E. coli* transformed with plasmids carrying individual A or B subunit genes or operon fusions were disrupted by sonication, and the proteins in the resulting lysates were labeled with ^{125}I as previously described (24). The iodinated proteins were immunoprecipitated with monoclonal antibody BC5 to the SLT-II B subunit (mouse ascitic fluid) as described by Downes et al. (5). The immunoprecipitates were analyzed on sodium dodecyl sulfate–15% polyacrylamide gels (14).

RESULTS

Plasmids carrying the individual A and B subunit genes for Shiga toxin/SLT-I, SLT-II, and SLT-IIv were constructed (Fig. 2). To permit the stable cotransformation of host bacteria, the A subunit genes of *stx*/*slt*-I, *slt*-II, and *slt*-IIv were cloned into the plasmid vector pACYC184, which has a P15A replicon (2), and the B subunit genes of the SLTs were cloned into either plasmid vector pBR328 (3) or expression vector pBluescript SK (Stratagene), both of which have a ColE1 replicon. These vectors also had different antibiotic resistance determinants so that positive selection for appropriate cotransformants could be maintained.

E. coli HB101 was cotransformed with plasmids carrying the individual A and B subunit genes from *stx*/*slt*-I, *slt*-II, and *slt*-IIv in all possible combinations. Sonic lysates and culture supernatants of the transformants were tested for cytotoxicity on Vero and HeLa cells to determine the levels of cell-associated and extracellular cytotoxin. In control experiments with *E. coli* HB101 transformed with each of the individual plasmids encoding one specific A or B subunit, no cytotoxicity was detected above the levels produced by

TABLE 2. Complementation studies: cytotoxicity on Vero and HeLa cells from *E. coli* HB101 producing hybrid toxins

Toxin		Cytotoxicity for Vero cells			Cytotoxicity for HeLa cells		
A subunit	B subunit	Cell associated[a]	Extracellular[a]	% Extracellular[b]	Cell associated	Extracellular	% Extracellular
I	I	7.4	5.6	1	7.4	5.6	1
I	II	ND[c]	ND	ND	ND	ND	ND
I	IIv	ND	ND	ND	ND	ND	ND
II	I	6.4	3.6	<1	−6.4	3.6	<1
II	II	6.4	5.6	14	6.4	5.6	14
II	IIv	3.4	3.6	62	ND	ND	ND
IIv	I	6.4	3.6	<1	5.4	3.6	1.4
IIv	II	6.4	5.6	14	6.4	5.6	14
IIv	IIv	6.4	7.6	94	ND	ND	ND

[a] Cell associated, $\log_{10}(CD_{50}$ per pellet); extracellular, $\log_{10}(CD_{50}$/40 ml of supernatant).
[b] % Extracellular (extracellular/[cell associated plus extracellular]) × 100.
[c] ND, No cytotoxicity detected above the levels produced by *E. coli* HB101.

E. coli HB101 alone (data not shown). The *E. coli* HB101 strains containing pairs of plasmids that encoded homologous A and B toxin subunits (Table 2) produced as much or more cytotoxin than did control HB101 strains containing the corresponding, intact *slt* operons on single plasmids (compare data in Table 2 to pMJ153, pMJ330, and pDLW5.321 in Table 3). These findings indicated that the A and B subunits produced by bacteria containing the subclones were biologically active and could associate in vivo to form functional holotoxins that were distributed between cell-associated and extracellular compartments in the same way as the native toxins.

The results of complementation tests with heterologous A and B subunit genes depended on the specific genes that were tested. *E. coli* HB101 cotransformed with pDLW102 and pJN26 (*slt*-II A and *slt*-I B) produced approximately 2.5×10^6 CD_{50} of cytotoxin that was exclusively cell associated and active on both Vero cells and HeLa cells. *E. coli* cotransformed with pDLW102 and pDLW105 (*slt*-II A and *slt*-IIv B) produced only 6.5×10^3 CD_{50} of cytotoxin that was predominantly extracellular and was toxic only for Vero cells. *E. coli* cotransformed with pDLW104 and pJN26 (*slt*-IIv A and *slt*-I B) or pDLW104 and pDLW103 (*slt*-IIv A and *slt*-II B) produced 2.5×10^6 CD_{50} of cytotoxin that was active against both Vero and HeLa cells, and in both cases the toxin was predominantly cell associated. No detectable cytotoxin was produced by *E. coli* cotransformed with pDLW101 and pDLW103 (*slt*-I A and *slt*-II B) or pDLW101 and pDLW105 (*slt*-I A and *slt*-IIv B). Cytotoxins with the B subunit from SLT-IIv were predominantly extracellular, whereas toxins with the B subunit from SLT-I or SLT-II were cell associated (98 and 86%, respectively). The mechanism(s) by which cytotoxins with the B subunit from SLT-IIv were exported was not determined. These studies demonstrate that the B subunit determines the cytotoxic specificity and cellular localization of holotoxin.

Because of the possibility that the copy number of the individual plasmids expressing A or B subunits might be different, hybrid cytotoxins produced by operon fusions were studied to confirm the complementation data. Unlike the subunit complementation analyses, both the A and B subunit genes of the fused hybrid operons were transcriptionally regulated by the naturally occurring promoter 5′ to the A subunit gene. Sonic lysates and culture supernatants of *E. coli* HB101 transformed with pMJ153 (*stx*), pMJ330 (*slt*-II), pDLW5.321 (*slt*-IIv), or the six operon fusion plasmids were tested on HeLa and Vero cells to determine the levels of cell-associated and extracellular cytotoxin (Table 3). The cytotoxicity profiles of the hybrid toxins produced by the operon fusions were similar to those observed in the complementation studies. *E. coli* HB101 transformed with pFUS4 (*slt*-IIA and *stx* B) produced approximately 2.5×10^5 total CD_{50} (cell associated plus extracellular) for Vero and HeLa cells. *E. coli* transformed with pFUS2 (*slt*-II A and *slt*-IIv B) produced approximately 6.5×10^4 total CD_{50} for Vero cells but was not cytotoxic for HeLa cells. *E. coli* transformed with pFUS6 (*slt*-IIv A and *stx* B) produced approximately 2.5×10^5 total CD_{50} for Vero and HeLa cells. *E. coli* transformed with pFUS1 (*slt*-IIv A and *slt*-II B) produced approximately 2.9×10^4 total CD_{50} for Vero and HeLa cells. *E. coli* cotransformed with pFUS3 (*stx* A and *slt*-II B) or pFUS5 (*stx* A and *slt*-IIv B) did not produce detectable levels of cytotoxin. As shown in Table 3, any cytotoxin containing the B subunit SLT-I or SLT-II was localized predominantly in the cell-associated fractions, whereas cytotoxins containing SLT-IIv B subunits were predominantly extracellular.

Neutralization, dot blot, and immunoprecipitation tests were done to characterize the immunochemical properties of the hybrid toxins and to further analyze the reasons for the lack of complementation between the *stx*/*slt*-I A gene and the B genes of *slt*-II or *slt*-IIv. Polyclonal antisera against Shiga

TABLE 3. Operon fusion studies: cytotoxicity on Vero and HeLa cells from *E. coli* HB101 producing hybrid toxins

Plasmid	Toxin		Cytotoxicity for Vero cells			Cytotoxicity for HeLa cells		
	A subunit	B subunit	Cell associated[a]	Extracellular[a]	% Extracellular[b]	Cell associated	Extracellular	% Extracellular
pMJ153	Shiga	Shiga	7.4	5.6	2	7.4	5.6	2
pFUS3	Shiga	II	ND[c]	ND	ND	ND	ND	ND
pFUS5	Shiga	IIv	ND	ND	ND	ND	ND	ND
pFUS4	II	Shiga	5.4	3.6	2	5.4	3.6	2
pMJ330	II	II	6.4	5.6	14	5.4	4.6	14
pFUS2	II	IIv	4.4	4.6	62	ND	ND	<1
pFUS6	IIv	Shiga	5.4	3.6	2	5.4	ND	<!
pFUS1	IIv	II	4.4	3.6	14	4.4	ND	<1
pDLW5.321	IIv	IIv	4.4	4.6	62	ND	ND	ND

[a] Cell associated, $\log_{10}(CD_{50}$ per pellet); extracellular, $\log_{10}(CD_{50}$/40 ml of supernatant).
[b] % Extracellular (extracellular/[cell associated plus extracellular]) × 100.
[c] No cytotoxicity detected above the levels produced by *E. coli* HB101.

toxin neutralized the hybrid cytotoxins that contained the B subunit of Shiga toxin/SLT-I, and the hybrid cytotoxins containing the A or B subunits of SLT-II or SLT-IIv were neutralized by polyclonal anti-SLT-II serum (data not shown).

Dot blot immunoassays were done on cell extracts of the transformants which produced no detectable cytotoxic activity (Shiga/SLT-I A subunit gene with SLT-II or SLT-IIv B subunit gene) to determine whether the B subunit with its native epitope was produced. *E. coli* transformed with pDLW101 and pDLW103 (*slt*-I A and *slt*-II B), pDLW101 and pDLW105 (*slt*-I A and *slt*-IIv B), pFUS3 (operon fusion containing *stx* A and *slt*-II B), or pFUS5 (operon fusion containing *stx* A and *slt*-IIv A) produced immunoreactive B subunits, as assessed by this procedure. Immunoprecipitation studies with these strains demonstrated that the A subunit of Shiga toxin/SLT-I assembled with the heterologous B subunit of SLT-II or SLT-IIv, even though hybrid holotoxins containing these subunits were not cytotoxic. Polypeptides corresponding in molecular weights to both the processed Shiga toxin/SLT-I A subunit (M_r, 32,000) and the processed SLT-II or SLT-IIv B subunit (M_r, 7,000) were immunoprecipitated by monoclonal antibodies specific either for the SLT-I A or the SLT-II B subunit (results not shown). It should be noted that low-level cytotoxin(s) produced by some wild-type *E. coli* strains cannot be detected by either dot blot immunoassays or immunoprecipitation.

To test the possibility that some active toxin was formed but at levels below detection with our standard assays, sonic lysates of *E. coli* transformed with pDLW101 and pDLW103 (*slt*-I A and *slt*-II B), pDLW101 and pDLW105 (*slt*-I A and *slt*-IIv B), pFUS3 (operon fusion containing *stx* A and *slt*-II B), or pFUS5 (operon fusion containing *stx* A and *slt*-IIv A) were further concentrated and tested. When these strains were concentrated 250-fold by ammonium sulfate precipitation, low levels of cytotoxin were detected (results not shown). When lysates of the *E. coli* host strain alone were similarly concentrated, no cytotoxic activity was detected. Therefore, the Shiga toxin/SLT-I A subunit and SLT-IIB and SLT-IIv B subunit can assemble and form active hybrid toxins, but the cytotoxic activity associated with the *E. coli* producing the hybrid toxins was greatly reduced compared with homologous toxins.

DISCUSSION

The B subunits of Shiga toxin/SLT-I and SLT-II bind to a eucaryotic cell receptor Gb_3 (12, 16–18, 36). However, SLT-IIv does not bind to an analog of Gb_3 in an enzyme-linked immunosorbent assay (38), and, unlike Shiga toxin/SLT-I and SLT-II, SLT-IIv kills Vero but not HeLa cells (21). The results of this study demonstrated that the different cell specificity of SLT-IIv compared with Shiga toxin/SLT-I and SLT-II is dictated by the SLT-IIv B subunit. Regardless of the source of the A subunit, hybrid toxins composed of the Shiga toxin/SLT-I B or SLT-II B subunit were equally cytotoxic for both Vero and HeLa cells. In contrast, hybrid cytotoxins composed of any A subunit and the SLT-IIv B subunit had the same cytotoxicity profile as SLT-IIv holotoxin, i.e., they killed Vero cells with no detectable activity on HeLa cells.

Previous studies with *E. coli* strains lysogenic for SLT-I or SLT-II-converting coliphage demonstrated that SLT-I was almost completely cell associated, whereas SLT-II was more equally distributed between the cell-associated and extracellular fractions (35). In *E. coli* producing SLT-IIv, most of the cytotoxin was localized to the extracellular milieu in *E. coli* K12 transformed with a plasmid encoding SLT-IIv (see pDLW5.321 in Table 3). With hybrid toxins, those that contained the Shiga toxin/SLT-I B subunit were almost exclusively cell associated (90%), while those hybrid toxins that contained the SLT-II B subunit were predominantly cell associated (86%). In contrast, hybrid cytotoxins that contained the SLT-IIv B subunit were predominantly extracellular (62%). Therefore, as with cell specificity, localization of the cytotoxins in *E. coli* is dictated by the source of the B subunit.

All of the hybrid cytotoxins were neutralized by polyclonal antisera specific for either Shiga toxin/SLT-I or SLT-II. This observation is in contrast to a recent study by Ito et al. (11), who found that hybrid cytotoxins containing SLT-I and SLT-II components were not neutralized by polyclonal antisera. One explanation for this difference in neutralization results is that our antisera were raised against native holotoxin, whereas the antitoxins used by Ito et al. were raised against formalin-treated SLT-I or SLT-II. Perhaps the epitopes exposed in the hybrid toxins are better recognized by antisera to native toxin than by antisera raised against the formalin-treated toxin.

Ito et al. (11) also detected a hybrid toxin composed of the SLT-I A and SLT-II B subunits with cytotoxic activity similar to toxin composed of homologous subunits. We were able to detect association of the Shiga toxin/SLT-I A subunit with heterologous SLT-II or SLT-IIv B subunits in vivo, but such hybrid molecules were several orders of magnitude less toxic than Shiga toxin/SLT-I. It is not clear why the specific toxicity of the SLT-I A and SLT-II B molecule formed in vitro was significantly greater than that of hybrids formed in vivo in our experiments.

Some inconsistencies were apparent when levels of cytotoxicity were compared between homologous toxins formed by subunit complementation and operon fusions. For example, the total CD_{50} produced by transformants of the parental control plasmids was sometimes higher than transformants of the hybrid plasmids (e.g., compare pMJ330 to pFUS2 in Table 3). The reasons for these variations in levels of cytotoxicity are not clear but may reflect the way in which the active molecules are assembled. Also, the total cytotoxicity of hybrid toxin produced by subunit complementation was sometimes different than the levels produced by operon fusions which combined the same A and B subunits (i.e., compare the CD_{50} of the hybrid toxin composed of SLT-IIv A/SLT-II B subunit produced by complementation [Table 2] to pFUS1 [SLT-IIv A/SLT-II B; Table 3]). Explanations for the production of hybrid toxins with elevated cytotoxicity compared with the production of native toxins include a gene dosage effect (e.g., higher plasmid copy number carrying a subunit gene or hybrid operon) or the enhanced transcription of a subunit gene or hybrid operon from an extragenic vector promoter.

In summary, we used subunit complementation and operon fusions to create hybrid cytotoxins. We found that B subunit dictated the cytotoxic specificity and localization of the toxin within the bacterial cell. Once these hybrid toxins are purified, the levels and specific activities of these cytotoxins will be analyzed. We will then be able to assess whether the levels of cytotoxicity (i.e., moderate or high) can be attributed to a specific subunit.

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